

Brain-Derived Neurotrophic Factor Is More Highly Conserved in Structure and Function than Nerve Growth Factor During Vertebrate Evolution

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Abstract: Mammalian nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) are members of a protein family with perfectly conserved domains arranged around the cysteine residues thought to stabilize an invariant three-dimensional scaffold in addition to distinct sequence motifs that convey different neuronal functions. To study their structural and functional conservation during evolution, we have compared NGF and BDNF from a lower vertebrate, the teleost fish *Xiphophorus*, with the mammalian homologues. Genomic clones encoding fish NGF and BDNF were isolated by cross-hybridization using probes from the cloned mammalian factors. Fish NGF and BDNF were expressed by means of recombinant vaccinia viruses, purified, and their neuronal survival specificities for different classes of neurons were found to mirror those of the mammalian factors. The half-maximal survival concentration for chick sensory neurons was 60 pg/ml for both fish and mammalian purified recombinant BDNF. However, the activity of recombinant fish NGF on both chick sensory

and sympathetic neurons was 6 ng/ml, 75-fold lower than that of mouse NGF. The different functional conservation of NGF and BDNF is also reflected in their structures. The DNA-deduced amino acid sequences of processed mature fish NGF and BDNF showed, compared to mouse, 63% and 90% identity, respectively, indicating that NGF had reached an optimized structure later than BDNF. The retrograde extrapolation of these data indicates that NGF and BDNF evolved at strikingly different rates from a common ancestral gene about 600 million years ago. By RNA gel blot analysis NGF mRNA was detected during late embryonic development; BDNF was present in adult brain. **Key Words:** Brain-derived neurotrophic factor—Nerve growth factor—Fish—Recombinant protein expression—Neuronal survival. Götz R. et al. Brain-derived neurotrophic factor is more highly conserved in structure and function than nerve growth factor during vertebrate evolution. *J. Neurochem.* 59, 432–442 (1992).

The basic building blocks of the nervous system, the neurons, show remarkable morphological and functional similarities throughout the animal phyla. However, the regulatory mechanisms involved in the development of the nervous systems at different evolutionary levels show different characteristics. In invertebrates, the development of the nervous system is generally subjected to a rigid, genetically determined pattern of cell lineage (e.g., Sulston and Horvitz, 1977; Truman and Schwartz, 1982). In contrast, the development of the nervous system of vertebrates, particularly higher vertebrates, depends extensively on epigenetic mechanisms, i.e., interactions of neurons with other neurons and with nonneuronal cells (Ja-

cobson, 1991). It is a general principle that in vertebrates, in the early stages of ontogeny a surplus of neurons is produced and after the arrival of their axons in the target areas, the final number of neurons is determined by regulated, regionally different neuronal cell death. The extent of neuronal death can be increased experimentally by removing the corresponding target tissues; conversely, grafting extra target tissue allows more neurons to survive (reviewed by Cowan et al., 1984). Neurotrophic factors produced by these neuronal and nonneuronal target cells are the molecular mediators that regulate the extent of neuronal survival and determine the density of innervation (reviewed by Barde, 1989).

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Abbreviations used: BDNF, brain-derived neurotrophic factor; NGF, nerve growth factor; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

The best characterized neurotrophic molecule is nerve growth factor (NGF). It plays an important role in the regulation of the survival, differentiation, and maintenance of specific functions of the peripheral sympathetic nervous system and subpopulations of sensory neural crest-derived neurons (reviewed by Levi-Montalcini, 1987). More recently, NGF has also been demonstrated to support subpopulations of neurons in the CNS, in particular cholinergic neurons of the basal forebrain nuclei (reviewed by Thoenen et al., 1987; Whitemore and Seiger, 1987). The recent cloning of brain-derived neurotrophic factor (BDNF) (Leibrock et al., 1989), neurotrophin-3 (Hohn et al., 1990; Maisonpierre et al., 1990; Rosenthal et al., 1990; Ernfors et al., 1990; Kaisho et al., 1990; Jones and Reichardt, 1990), and neurotrophin-4 (Hallböök et al., 1991) revealed the existence of additional neurotrophic factors that are structurally related to NGF, and yet show, as far as established, distinctly different neuronal specificities and different patterns of regional distribution, cellular localization, and developmental expression (reviewed by Barde, 1989; Thoenen, 1991). So far the comparative information on the different members of the NGF gene family has been restricted to higher vertebrates. Recently, partial sequences (encoding ~42 amino acid residues) of neurotrophins of lower vertebrates have been determined (Hallböök et al., 1991). The comparative structure-function analysis of neurotrophic factors of lower vertebrates and mammals can be expected to reveal important new information provided such an analysis is performed in quantitative assay systems and with purified proteins. Moreover, the evolutionary analysis should provide information as to whether and when the different members of the gene family originated from a common ancestor.

As a first step toward this goal we investigated an organism at the stem of the vertebrate branch of the phylogenetic tree. Fish represent one of the oldest vertebrate groups. They diverged approximately 450 million years ago from the vertebrate stem. We have chosen the fish *Xiphophorus* to study the presence, biological significance, and evolutionary origin of NGF and BDNF. This small teleost species is currently one of the best characterized fish genetically and molecularly and, in addition, is easy to grow (reviewed by Adam et al., 1990).

We report here the cloning of NGF and BDNF from the platyfish *Xiphophorus maculatus* and the production and biological characterization of recombinant fish NGF and BDNF. The production and purification of recombinant fish NGF and BDNF permitted the determination of their neuronal survival specificities and their specific activities. In agreement with the highly conserved structure (90% amino acid identity) the biological activity of fish BDNF was identical to that of mammalian BDNF. In contrast, the specific activity of fish NGF analyzed in chick sensory and sympathetic neurons is considerably

lower than that of mouse NGF. This was reflected by a much lower (63%) structural conservation.

MATERIALS AND METHODS

Cloning of NGF and BDNF

A genomic library that had been prepared by inserting DNA from *Xiphophorus maculatus* (origin Rio Usumacinta, Mexico) partially digested with *Sau3A* into the *Bam*HI cloning site of the bacteriophage vector EMBL4 (Frischauf et al., 1983) was screened (Benton and Davis, 1977) under reduced stringency conditions with radioactively labeled DNA fragments corresponding to mature pig BDNF (Leibrock et al., 1989). The probe was labeled with [α - 32 P]dCTP as described by Rigby et al. (1977) and Feinberg and Vogelstein (1983). The conditions for hybridization had been established on Southern blots (Southern, 1975) of genomic fish DNA and were 5 \times SSC (1 \times SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.2), 40% formamide at 42°C with washes in 2 \times SSC at 60°C. Clones were plaque purified and their DNA prepared and mapped with restriction endonucleases according to standard protocols (Maniatis et al., 1982). The 1.8- and 1.3-kbp *Eco*RI fragments hybridizing with the BDNF probe were subcloned into pUC13 and M13mp18 vectors (Norlander et al., 1983) for further analysis.

To clone the fish NGF, genomic DNA fragments in the size range 2.6–3.3 kbp separated by agarose gel electrophoresis were purified (Vogelstein and Gillespie, 1979), ligated into the vector λ gt10 (Huynh et al., 1985), and packaged into phage particles as specified by the manufacturer of the packaging kit (Amersham). A positive clone (A5.2) was isolated as described above and its insert was subcloned into the plasmid vector pT7T3 18U (Pharmacia).

DNA sequencing

DNA sequencing was done on both strands according to the chain termination method (Sanger et al., 1977) with 35 S-dATP and T7 DNA polymerase using the instructions of the supplier of the enzyme (Pharmacia). We used both double- and single-stranded templates that had been constructed by subcloning appropriate restriction fragments and by generating nested deletions with exonuclease III as described by Henikoff (1984). As primers M13 universal primer, M13 reverse primer, and insert-specific primers were employed. Oligonucleotides were synthesized by an Applied Biosystems automated DNA synthesizer. The sequences were analyzed with the UWGCG programs (Devereux et al., 1984).

Expression of neurotrophic factors

Two recombination plasmids were constructed that contained the fish NGF or BDNF coding sequences. For NGF expression, a 1,087-bp *Hind*III–*Dra*I fragment (see Fig. 1) was inserted into the vector 11kd6-131 linearized with *Hind*III and *Eco*RV. To express BDNF, the 1,304-bp *Eco*RI fragment encoding BDNF in the λ phage clone B14 (see Fig. 1) was cloned in the correct orientation for protein expression into the vector 11k-Ata 18 (Stunnenberg et al., 1988). The vector 11kd6-131 is similar to 11k-Ata 18 except for the polylinker. Vaccinia recombinant virus was generated by transfection with plasmid DNA of human TK⁻ 143 cells (Rhim et al., 1975) infected with wild-type virus (strain WR) and subsequent selection of TK⁻ virus as described by Mackett et al. (1984). Recombinant virus was differentiated

from spontaneous TK⁻ virus by polymerase chain reaction assays (Saiki et al., 1988) with oligonucleotide primers specific for the NGF or BDNF inserts. For protein production, rabbit kidney cells (RK₁₃ cell line, ATCC number CCL 37) grown as monolayers in plastic culture flasks were infected with recombinant virus and after a 6-h growth period in Dulbecco's modified Eagle's medium containing 5% fetal calf serum, medium was removed and cells were washed with phosphate-buffered saline (PBS). Serum-free medium was added and after incubation for 24–36 h, conditioned medium was harvested. After cells had been removed by centrifugation, the conditioned medium was pumped over a 4-ml (bed volume) controlled pore glass column essentially as described by Eichner et al. (1989); washed with 200 ml PBS; and eluted with 20 ml of 50% acetonitrile, 100 mM NaCl, and 100 mM acetic acid. Purification was achieved by reversed-phase HPLC (Aquapore RP-300 column, 2.1 × 22 mm, Applied Biosystems). The purification was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Band intensities on gels were quantitated using an Ultrascan laser densitometer (Pharmacia-LKB Instruments) with known amounts of a lysozyme standard for calibration. Protein concentrations in conditioned medium were determined by the method of Bradford (1976). The neurotrophic activity was assayed according to the method of Lindsay et al. (1985).

Northern (RNA) analysis

RNA isolation following the LiCl/urea procedure was performed as described previously by Mäueler et al. (1988). Twenty micrograms of total RNA was separated by electrophoresis on a 1.2% agarose gel in the presence of formaldehyde and then transferred to Hybond N nylon membranes

(Amersham). RNA standards (0.24- to 9.5-kb RNA ladder, BRL) were run in parallel for sizing. After UV fixation, the membrane-bound RNA was stained with methylene blue (Khandjian, 1986) for the exact quantitation of the amount of RNA transferred. Prehybridization and hybridization were performed as previously described by Mäueler et al. (1988) with final washes at 65°C in 0.1 × SSC, 1% SDS. The 0.6-kbp *Sma*I–*Pst*I fragment of the BDNF gene (see Fig. 1) was ³²P labeled with the random priming protocol (Feinberg and Vogelstein, 1983) and used as probe. The NGF antisense riboprobe (complementary to nucleotide positions 258–611; see Fig. 2) was transcribed from plasmid 1627 linearized with *Hind*III using T3 RNA polymerase following the protocol supplied by the manufacturer (Stratagene). Plasmid 1627 carries a 354-bp *Hind*III–*Eco*47III NGF subfragment ligated into the vector pT7T3-19 (Pharmacia) linearized with *Hind*III and *Sma*I. For the removal of the probe, filters were incubated in 1% SDS at 80°C for 20 min, and subjected to rehybridization after autoradiographic control.

Nucleotide sequence accession number

These sequence data are available from EMBL/GenBank/DBJ under accession numbers X59941 (fish NGF) and X59942 (fish BDNF).

RESULTS

Cloning of fish NGF and BDNF

Southern blots probed under low-stringency conditions with radioactively labeled fragments that corresponded to either mouse NGF (Scott et al., 1983) or

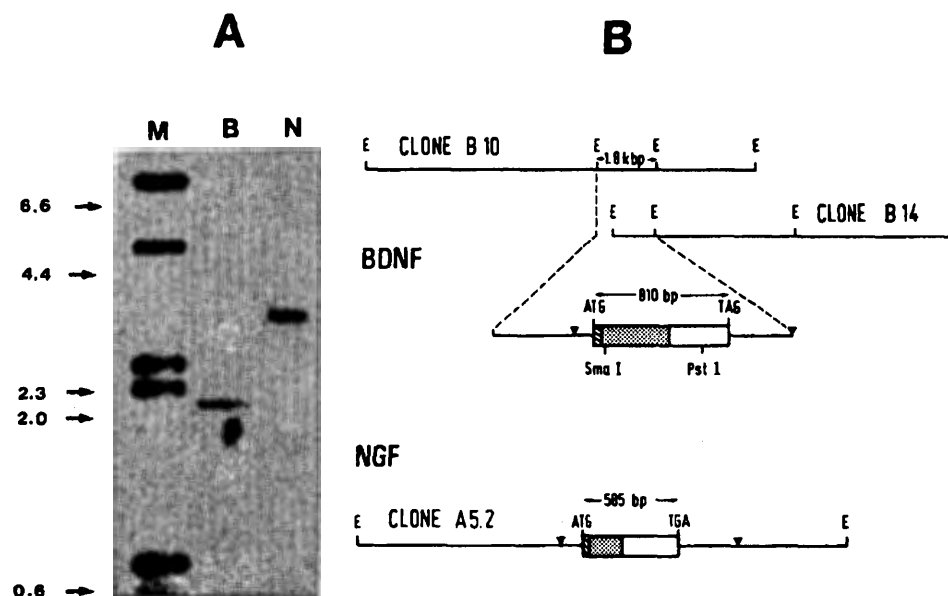


FIG. 1. Cloning of the NGF and BDNF genes of *Xiphophorus*. **A:** Detection of fish NGF and BDNF sequences in Southern blots of *Xiphophorus* DNA digested with *Eco*RI and probed under reduced stringency conditions (as specified in Materials and Methods) with mouse NGF (lane N) or pig BDNF (lane B). Size markers were ³²P labeled λ *Hind*III fragments; the sizes are indicated in kbp (lane M). **B:** A map depicting the *Eco*RI (E) inserts of the BDNF and NGF clones is shown. For BDNF, an expanded representation of the genomic region whose sequence has been determined is given. Start codons, signal peptide regions (hatched segment), pro-regions (stippled box), mature regions (open box), and stop codons are indicated. The filled arrowheads mark the subfragments cloned for the expression of neurotrophic factors.

pig BDNF (Leibrock et al., 1989) revealed a specific hybridizing band for each probe in the fish genome (Fig. 1A). Because the precursor proteins of NGF and BDNF of higher vertebrates are encoded in single exons (Ullrich et al., 1983; Selby et al., 1987a; Leibrock et al., 1989) and because the intron/exon arrangements of homologous genes in fish and higher vertebrates have so far been found to be conserved (e.g., see Adam et al., 1991; Hannig et al., 1991), we isolated clones from genomic libraries. The 2.9-kbp *EcoRI* fragment identified with the NGF probe in the

Southern blot was cloned from a size-selected genomic library (Fig. 1B). With the BDNF probe we identified a series of overlapping clones from a representative library that contained the hybridizing 1.8-kbp *EcoRI* fragment (Fig. 1B).

Strikingly different conservation of NGF and BDNF structures

The identification of the isolated NGF gene is based on the following features: it encodes a protein with a molecular mass of 21.6 kDa (194 amino acid resi-

NGF

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1  ACAACAGTGTGTTTTCACAACACATACAAGAGCTGTTCTCTTTATTTGGGTGTTTTGATTGTGTTGAACAGTGTGTTTCGCTCTCTCATTGGTTATCTTAGATCGTGTGCC 120
      * Q L F S L F S L V I L D R V P
121  ATGAGGTCATCCATGCTGGTCTGTTCTCATCTTCAGTGCCCAAGGCTGTGGCCCCCATATAGGGGCTCTGTGCAGCGTCACAACAGCACAAAGAGGACCCACCTCCATCCCACT 240
1  [M] R S S N L V L F L I F S A Q A V A P I I G V L C S V T T A Q Q D H P T S I P T 40
241  GTGGACCCCAAGCTCTTCAATAAGCGCCGCCACCTCTCACCAGGGTCTTTTTCAGCTCACAGCCCCCGATGCGAGCCAGCAGGGGGACAGGGTGTTCAGCAGGAGACTCGAAGGCAG 360
41  V D P K L F N K R R H L S P R V L F S S Q P P D A E P A G G Q G V S R R T R R Q 80
361  CCTCAGCACCAGGGGGTGTACTCGGTGTGTGAGAGTGTAGTGTCTGGGTGGGCAACAAACCAAGCCACAGACATCTCAGGCAAGAGGTGACAGTGTCCCATATGTGAACATAAC 480
81  P Q H R G V Y S V C E S V S V W V G N K T K A T D I S G K E V T V L P Y V N I N 120
481  AATGTTAAGAAGAACAGTATTTCTTTGAGACAGGTGTACAGCCCTCCATCTGGAGGCTCAAGATGTTTAGGAATTGACGCAAGGCACTGGAACTCCCATGTGCACTACCTCGACACT 600
121  N V K K K Q Y F F E T T C H S P P S G G S R C L G I D A R H W N S H C T N S H T 160
601  TTCGTCCGAGCCTCACTTTCATCCGAGAACAGGTGGCTGGAGGCTCATTCGATCAAGTGGCTGTGTGTGCTGCTCAGCCGCAATCGTGGCAGTGAAGACTCACCCTATAGA 720
161  F V R A L T S S E N Q V A W R L I R I N V A C V C V L S R K S W Q H *

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BDNF

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1  ATTACGTGATGATTATGATCAAGAGGGGCTAGCTTTGGTAGAACTACTCTTCACTAACCACCTTGTCTCTTCCCTCTTCTGTTCTCCCTCCAGTCCACAGGTTAGAGAGTG 120
      * C I N D Q E G A S F G R N T L S L T T L F S S L F L F S L Q F H Q V R R V
121  ATGACCATCCTGTTCTTACTATGTTATTTATCATCTTCAGTGCATGAGAGCTGCSCCCCTGAGAGACGCCCGGCGATGCGGGGCTATGAGCGGAGGCTACCTGGGCGTGTCTGCG 240
1  [M] T I L F L T M V I S Y F S C H R A A P L R D A P G M R G H W T E G Y L G A A A 40
241  ACGGCCCCCGAGGCCATGGGATCCACAGAGTGGGGGCGGGGCGGCGAGGAGCTCCCTCGCTCAGACACATTGAGCAGGTGATAGAGGAGCTGCTGGAAGTGGAGGT 360
41  T A P R G H G T P Q S G G G P G Q R E E L P S L T D T F E Q V I E E L L E V E G 80
361  GAGGCGGCGCAGTGGGACAGGGGGGCGACAAGAGTCAGGGAGGTGGGGGCCCTGCGCCCTGGCCACCGCAGAGGCCAATGATGTCGATCTGTACAACTCGCGGGTGTATGATCAGCAAC 480
81  E A A H V G Q G A D K S Q G G G G P S P V A T A E A N D V D L Y N S R V M I S N 120
481  CAAGTGCCTTTGGAGCGCCCTTGTCTTCTCTGAGGAAATACAAAACATATCTGGATGCTGCGAATGTCCATGAGGGTGGCGGCGACTCCGACCCCTCGCGGCTGGAGAGCTC 600
121  Q V P L E P P L L F L L E E Y K N Y L D A A N M S M R V R R H S D P S R R G E L 160
601  AGTGTGTGTGACAGTATTAGCCAGTGGGTGACAGCTGTGGATAAAAGACGGCGATAGACATGTCTGGGAGACAGTAACCGTCATGGAGAAGGTCCCTGTCCCAATGGCCAACTGAAG 720
161  S V C D S I S Q W V T A V D K K T A I D M S G Q T V T V M E K V P V P N G Q L K 200
721  CAATACTTTTATGAGACCAATGCAACCCCATGGGTACACAAGGACGGCTGCAGAGGAATAGACAAGCGGCAATTATACATCCCAATGACAGGACACCCAGTCTACGTGCGAGCGCTC 840
201  Q Y F Y E T K - C N P M G Y T K D G C R G I D K R H Y T S Q C R T T Q S Y V R A L 240
841  ACCATGATAGACAAAAGAGAGTGTGGTGGCGTTTATAAGGATAGACACTTCATGTGTATGACATTGACCATTAAGAGAGGAGATAGTGTATAAAATGATAGATTTTATTGAAGAG 960
241  T M D S K K K I G W R F I R I D T S C V C T L T I K R G R *

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FIG. 2. Fish NGF and BDNF gene and amino acid sequences. The open reading frame of the NGF and BDNF genes is shown; the starting methionine is boxed. Proteolytic cleavage to release the mature factor is marked with a filled arrowhead; these data are based on aminoterminal sequencing of the recombinant proteins (see Results). Predicted signal peptidase cleavage is indicated with an open arrowhead, potential sites for N-linked glycosylation are underlined, and sequences with dibasic motifs are double underlined.

dues), the carboxyl terminal 115 amino acids (the predicted mature form of fish NGF) displaying 63% identity to mature mouse NGF (Figs. 2 and 3; Table 1). The identification as fish NGF is based on the lower sequence identity to mouse BDNF (43%) and mouse neurotrophin-3 (48%) and was substantiated further by the activity of the recombinant protein (see below). Moreover, a different band hybridized in the Southern blot of fish DNA with the mouse NT-3 probe (data not shown). The initiating AUG is in accordance with empirical rules established for vertebrate mRNAs (Kozak, 1987). The N-terminal of the specified protein fulfills the characteristics of a signal peptide (Von Heijne, 1986). The pro-region of fish NGF shows a significant homology to the pro-segment of NGF from other species (e.g., 51% identity to the mouse sequence) but its length of 60 amino acids is considerably shorter than in other species (e.g., 103 amino acids in the mouse; for sequences see Schwarz et al., 1989). The predicted (and in recombinant molecules demonstrated) N-terminal of the mature protein is located 11 residues from the first cysteine (see Figs. 2 and 3). The cleavage signal corresponds to that in all other NGFs and also neurotrophins analysed so far, the (-4 to -1)-consensus being R-X-K/R-R.

The fish BDNF gene sequence contains an open-reading frame of 269 amino acid codons (Fig. 2) presumed to encode the BDNF precursor (deduced molecular mass, 29.7 kDa). The predicted organization of the fish BDNF precursor strikingly resembles that of the mammalian precursors in size and sequence. The mature fish BDNF which, like its mammalian counterparts, also consists of 119 amino acids (residues 151–269 in Fig. 2), is 90% identical to that of mouse (Figs. 2 and 3; Table 1). The N-terminal sequence (residues 1–18 in Fig. 2) serves as a signal peptide and shows a remarkable conservation to the mouse signal sequence (89% identity). The pro-region adjacent to the mature protein has a region of 43 residues with 88% identity to the mouse protein, the cleavage site (R-V-R-R) of the precursor being identical.

All available sequences were used to calculate the genetic distances for the orthologous gene pairs, corrected for "unobservable" changes according to Dayhoff et al. (1978) and the divergence rates (Table 1). This analysis revealed that the BDNF structure has diverged only very slowly between fish and mammals. In contrast, NGF has evolved at an approximately fivefold higher rate. Later in evolution, the sequence drift for NGF protein has decreased as indicated by

NGF-fish	Q P Q H R G V Y S V C E S V S V W V G - - N K T K A T D I S G K E	
NGF-cobra	E D H P V H N L G E H S V C D S V S A M V - - T K T T A T D I K G N T	
NGF-chick	T A H P V L H R G E F S V C D S V S M W V G - - D K T T A T D I K G K E	
NGF-mouse	S S T H P V F H M G E F S V C D S V S V W V G - - D K T T A T D I K G K E	
NGF-bovine	S S S H P V F H R G E F S V C D S I S V W V G - - D K T T A T D I K G K E	
NGF-human	S S S H P I F H R G E F S V C D S V S V W V G - - D K T T A T D I K G K E	
NGF-con	S S S H P I F H R G E F S V C D S V S V W V G - - D K T T A T D I K G K E	
BDNF-con	H S D P R R G E L S V C D S I S M V T A D K K T A D M S G T	
BDNF-fish	H S D P S R R G E L S V C D S I S Q W V T A V D K K T A I D M S G Q T	
BDNF-mouse	H S D P A R R G E L S V C D S I S E W V T A A D K K T A V D M S G G T	

NGF-fish	V T V L P Y V N I N H V K K K Q Y F F E T T C H S P P S G G S R C L G I D	
NGF-cobra	V T V M E N V N L D N K V Y K Q Y F F E T T C K N P N P E P S G C R G I D	
NGF-chick	V T V L G E V N I N H V F K Q Y F F E T T C R A S R P P V S S G C R G I D	
NGF-mouse	V T V L A E V N I N H S V F K Q Y F F E T T C R D P N P V S S G C R G I D	
NGF-bovine	V M V L G E V N I N H S V F K Q Y F F E T T C R D P N P V S S G C R G I D	
NGF-human	V N V L G E V N I N H S V F K Q Y F F E T T C R D P N P V S S G C R G I D	
NGF-con	V V V N H M D Y F F E T T C S C G I D	
BDNF-con	V T V E K V P V G O L K Q Y F Y E T K C N P M G Y T K G C R G I D	
BDNF-fish	V T V M E K V P V P N G O L K Q Y F Y E T K C N P M G Y T K D G C R G I D	
BDNF-mouse	V T V L E K V P V S K G Q L K Q Y F Y E T K C N P M G Y T K E G C R G I D	

NGF-fish	A R H W N S H C T T N S H T F V R A L T S S E N Q V A - W R L I R I N V A C	
NGF-cobra	S S H W N S Y C T E T D T F I K A L T M E G N Q A S - W R F I R I E T A C	
NGF-chick	A K H W N S Y C T T T H T F V K A L T M E G K Q A A - W R F I R I D T A C	
NGF-mouse	S K H W N S Y C T T T H T F V K A L T T D E K Q A A - W R F I R I D T A C	
NGF-bovine	A K H W N S Y C T T T H T F V K A L T M D G K Q A A - W R F I R I D T A C	
NGF-human	S K H W N S Y C T T T H T F V K A L T M D G K Q A A - W R F I R I D T A C	
NGF-con	H W N S C T T F A L T Q W R I R I A C	
BDNF-con	K R H S O C R T T O S Y V R A L T H D S K K I G W R F I R I D T S C	
BDNF-fish	K R H Y T S Q C R T T Q S Y V R A L T H D S K K I G W R F I R I D T S C	
BDNF-mouse	K R H W N S Q C R T T Q S Y V R A L T H D S K K R I G W R F I R I D T S C	

NGF-fish	V C V L S R K S W Q H	(116)
NGF-cobra	V C V I T K K K G M	(116)
NGF-chick	V C V L S R K S G R P	(116)
NGF-mouse	V C V L S R K A T R	(116)
NGF-bovine	V C V L S R K T G Q R A	(120)
NGF-human	V C V L S R K A V R R A	(120)
NGF-con	V C V K	
BDNF-con	V C T L T I K R G R	(110)
BDNF-fish	V C T L T I K R G R	(110)
BDNF-mouse	V C T L T I K R G R	(110)

FIG. 3. Evolutionary comparison of neurotrophic factors. The amino acid sequences of fish NGF and BDNF are aligned with those of cobra NGF (Selby et al., 1987b; Oda et al., 1989), chicken NGF (Ebendal et al., 1986; Meier et al., 1986; Wion et al., 1986), bovine NGF (Meier et al., 1986), mouse NGF (Angeletti and Bradshaw, 1971; Scott et al., 1983), human NGF (Ullrich et al., 1983), and mouse BDNF (Hofer et al., 1990). Gaps are indicated by dashes to obtain the best homologies. A consensus ("con") for NGF and BDNF is given (underlined); residues identical in all sequences are indicated with an asterisk. The cysteines are marked by squares above the sequence. Residues that are identical in all NGFs but not conserved in BDNF are indicated (+) and residues in fish NGF that differ from the chick/mammals consensus are marked (:). The presumed lengths of the mature neurotrophic proteins are shown at the end of the sequences.

TABLE 1. Conservation of NGF and BDNF protein/gene structures

	Percent difference	PAM ^a	Evolutionary rate ^b
BDNF, fish/mouse	10/18	10.7/20.5	1.2/2.5
NGF, fish/cobra	48/37	74/51	8.2/5.7
NGF, fish/chick	36/33	48/43	5.3/4.8
NGF, fish/mouse	37/31	52/40	5.8/4.4
NGF, fish/bull	36/32	48/41	5.3/4.6
NGF, fish/man	37/33	50/43	5.6/4.8
NGF, chick/mouse	16/20	17.9/23	3.6/4.6
NGF, chick/bull	10/15	10.7/16.5	2.1/3.3
NGF, chick/man	12/17	13.0/19	2.6/3.8

^a Evolutionary distance in amino acid PAMs and nucleotide PAMs, respectively [accepted point mutations per 100 residues, according to Dayhoff et al. (1978)].

^b PAM per 100 million years.

the comparison between chick and mammals (Table 1). The retrograde extrapolation of the genetic distances and evolutionary rates given in Table 1 indicates that NGF and BDNF have arisen from an ancestral gene by a gene duplication event approximately 600 million years ago.

The NGF and BDNF proteins are colinear and show several domains of strictly conserved amino acids predominantly around the cysteine residues (Fig. 3). One deletion of two residues near the N-terminal of NGF and another one near the C-terminus becomes apparent from this comparison. The consensus amino acids of BDNF amount to 107, whereas for NGF, the level of conservation is less pronounced: 71 residues are identical between fish and mouse and 55 are identical in all species.

To obtain additional information of sequence motifs located at the surface of the neurotrophins, their hydrophilicity profiles were determined using the algorithm of Hopp and Woods (Hopp and Woods, 1981). Whereas the plots of fish and mammalian BDNF are very similar, marked differences become apparent when the NGF plots of fish and higher vertebrates are compared (Fig. 4).

Transcriptional analyses of the NGF and BDNF genes

In northern blots (Fig. 5) the expression of the BDNF gene (size of transcript 1.8 kb) was restricted to adult brain; no signal could be detected in RNA from eye or liver of adult fish. No transcript could be detected following reprobing of the same filter with the NGF probe (not shown). A 3.6-kb message specific for NGF was detected during late organogenesis in embryos between 7 and 17 days old [embryonic stages 16–24 according to Tavalga (1949)] but not in younger embryos or in fish after birth; no BDNF transcript could be detected in these developmental stages (not shown).

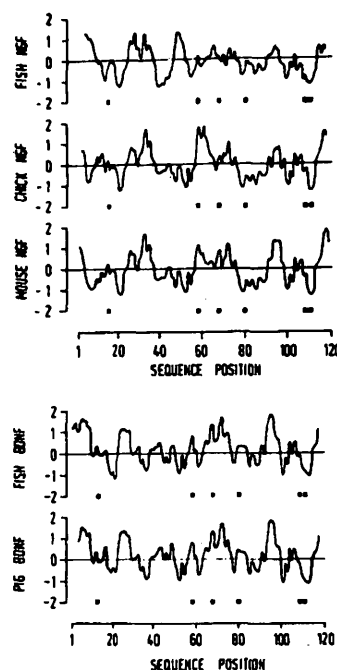


FIG. 4. Comparative hydrophilicity plots of fish and higher vertebrate neurotrophic factors. The average hydrophilicity values (Hopp and Woods, 1981) were calculated over a hexapeptide. The segments of NGF and BDNF predicted to be localized on the (hydrophilic) surface are on the positive end of the y-axis. The squares show the position of cysteines.

Expression and purification of fish NGF and BDNF

Because the NGF and BDNF structures have evolved at strikingly different rates we wanted to compare the specific biological activities of the fish pro-

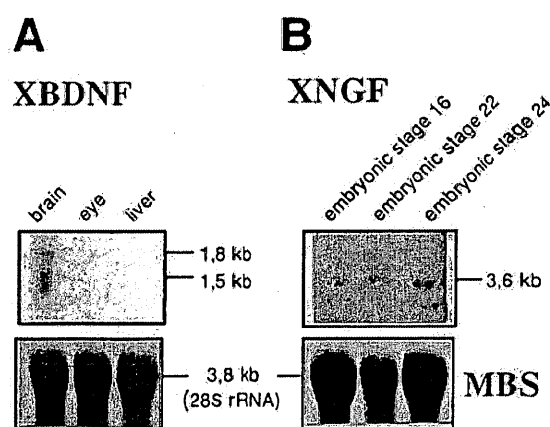
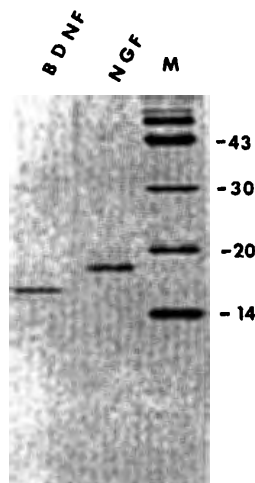


FIG. 5. Expression of (A) BDNF and (B) NGF in *Xiphophorus*. Northern blot hybridization of total RNA (20 μ g) isolated from brain, eye, and liver of adult fish or from embryos of the indicated stages; on the right, the transcript sizes are indicated in kb. Below, methylene blue stain (MBS) for quantitation of filter-bound RNA.

FIG. 6. SDS-PAGE of purified fish NGF and BDNF. SDS-PAGE was carried out with reduced samples after HPLC chromatography on an 18% (wt/vol) polyacrylamide gel and stained with Coomassie Blue. Molecular mass markers are indicated in kilodaltons in lane M and are from top to bottom ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and α -lactalbumin.



teins to their mammalian homologues. To obtain recombinant fish NGF and BDNF proteins, vaccinia virus expression vectors with the NGF or BDNF genes inserted into their genome were constructed. The conditioned medium harvested from vaccinia virus-infected cells contained at least 200 ng/ml of recombinant protein (~1% of the total protein). The recombinant proteins were purified in a two-step procedure on controlled-pore glass followed by reversed-phase HPLC; the SDS-polyacrylamide gel of fish NGF and fish BDNF is shown in Fig. 6. The BDNF preparation showed a single band of ~15 kDa which is close to the value of 13.6 kDa predicted from the DNA sequence; its N-terminal sequence was determined as $\text{NH}_2\text{-H-S-D-P-S}$. The electrophoretic mobility of recombinant fish NGF was somewhat lower but aminoterminal sequencing yielded the sequence

$\text{NH}_2\text{-Q-P-Q-H-R}$. Thus proteolytic processing had occurred as predicted at the carboxyl side of the sequence motif R-T-R-R (Fig. 2).

Biological activity of fish NGF and BDNF

The survival effect of 1.5 ng/ml fish BDNF on chick embryonic sensory neurons was maximal, identical to that obtained with the same concentration of natural (purified from pig brain) or recombinant mammalian BDNF. In contrast, fish NGF at 1.5 ng/ml showed a detectable but by no means maximal survival effect (Fig. 7). However, when fish NGF was used at 25 ng/ml, the same level of survival was obtained as with maximal concentrations of mouse NGF. Furthermore, the activities of fish NGF and fish BDNF (both added at supramaximal concentrations) were additive, indicating that the factors acted on different subpopulations of neurons. The survival and neurite outgrowth promoting activity of fish NGF on chick sympathetic neurons (which do not respond to BDNF) was similar to the findings on sensory neurons in that a concentration of ≥ 25 ng/ml was needed to obtain maximal survival values (Fig. 7). Furthermore, fish BDNF but not fish NGF supported the survival of nodose neurons (Fig. 7).

A dose-response curve of the survival of sensory neurons in response to fish NGF and fish BDNF is shown in Fig. 8. Half-maximal effects were obtained with 6 ng/ml of fish NGF; this specific activity is ~75-fold lower than that of mouse NGF (Edgar and Thoenen, 1982) or bovine NGF (Harper et al., 1983). Very similar results were obtained with chick sympathetic neurons (data not shown). The biological activity of fish BDNF assayed on sensory dorsal root ganglion neurons (half-maximal survival with 60 pg/ml; Fig. 8) was undistinguishable from that of the mam-

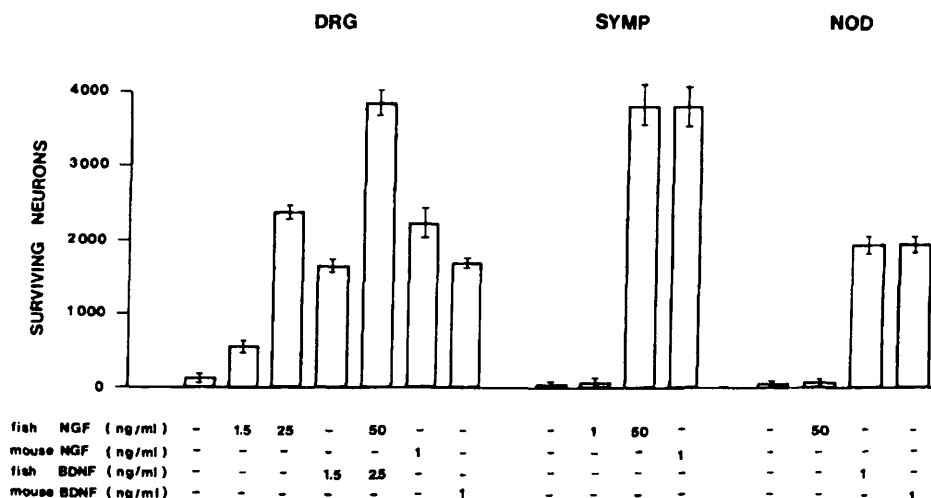


FIG. 7. Specificity of the survival activity of fish NGF and BDNF. Bar charts showing the survival response of sensory neurons prepared from dorsal root ganglia (DRG) of 8-day-old chick embryos (E8), and sympathetic (SYMP, E11) and nodose ganglion (NOD, E8) neurons. Six thousand neurons were plated on laminin/polyornithine substrate and the surviving neurons were counted after 1 day in culture.

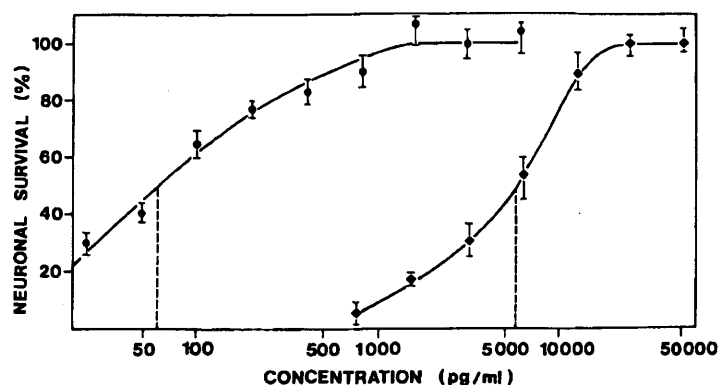


FIG. 8. Dose dependency of the survival activity of fish NGF and fish BDNF. Dose-response curves for the survival of sensory neurons prepared from the dorsal root ganglia of chick in response to fish NGF (◆) or fish BDNF (●), maximal survival being defined as 100%. Six thousand neurons were plated on laminin/polyornithine substrate and the surviving neurons were counted after 1 day in culture. The broken vertical lines show the concentrations of 60 pg/ml of BDNF and 6 ng/ml of NGF for half-maximal survival.

malian factor purified from pig brain (Rodriguez-Tébar and Barde, 1988) or produced using recombinant vaccinia virus. The biological activity of fish BDNF assayed on chick sensory neurons (half-maximal at 60 pg/ml $\approx 2 \times 10^{-12}$ M) correlates with high-affinity receptors.

DISCUSSION

Structure-function relationship

The amino acid sequences of all NGFs and BDNFs analyzed so far, ranging from fish to mammals, are defined by six strictly conserved cysteines (Fig. 3). These cysteines form three intramolecular disulfide bridges whose positions have been determined for mouse NGF (Angeletti and Bradshaw, 1971) and are presumed to be conserved in all other NGF homologs. Besides the 6 cysteines, 34 additional residues are identical in all NGF and BDNF sequences, suggesting that these residues are essential for the correct folding of the molecules, the stabilization of their three-dimensional structure, and the maintenance of their biological activity.

Conversely, there are domains that are different from each other and that determine the different spectra of neuronal specificity of NGF and BDNF (see Barde, 1989; Thoenen, 1991). Fifteen amino acid residues are proposed to belong to this second class (Fig. 3), as they are conserved in all NGFs but different in all BDNFs. They are clustered in five or six domains, indicating that several domains (rather than a single one) determine neuronal specificity. Interestingly, the hydropathy plots of NGF and BDNF structures (Fig. 4) differ in these domains due to nonconservative amino acid replacements. This interpretation is strongly supported by a recent study in which the neuronal specificities of NGF-BDNF chimeric molecules were compared and that demonstrated that more than one domain is responsible for the corresponding neuronal specificity (Ibáñez et al., 1991). However, because the chimeric proteins were not purified and the biological activity was determined in a fiber-out-

growth assay using ganglion explants, no information on changes in potency of the chimeric proteins was deduced.

The comparison of the sequences of NGFs of higher vertebrates demonstrated that the replacements (which did not result in changes of the specific activity) were generally located in hydrophilic regions (Meier et al., 1986) representing potential immunogenic epitopes and explaining the limited immunological crossreactivity between the different NGFs (Harper et al., 1983). In contrast to the hydropathy plots of BDNF, which are indistinguishable from fish to mammals, the fish NGF plot shows a different profile as compared to higher vertebrates (Fig. 4). Twenty-six residues in fish NGF are different from the chick/mammal NGF consensus (see Fig. 3) and (some) might be responsible for the observed 75-fold lower specific activity of fish NGF on chick sensory and sympathetic neurons as compared to the effects of mouse NGF. Nevertheless, the spectrum of neuronal specificity of fish NGF is preserved. It is noteworthy that several of the changed residues are predicted to be located on the protein surface, e.g., in the regions of the hydrophilic peaks at positions 33 and 47 (Fig. 4). Interestingly, the determination of the crystal structure of mouse NGF revealed that these hydrophilic peaks are surface-exposed β -hairpin loops (McDonald et al., 1991). According to the more rapid evolutionary changes from fish to bird, the hydropathy plot of snake NGF shows, in comparison to that of higher vertebrates, some differences (not shown) which are also reflected by a low specific activity as determined in the ganglion explant assay (Server et al., 1976).

Evolution of the neurotrophic gene family

If the evolutionary divergence rates of NGF and BDNF are plotted against a common time scale, the gene duplication event is estimated to have happened approximately 600 million years ago. This is the time when the ancestral vertebrates first appeared in the fossil record. The gene duplication event of the common, probably BDNF-like ancestor is estimated to

have occurred significantly earlier than the divergence of the fish lineage but later than the divergence of the insects from the phylogenetic tree. The new second copy was then free of selective pressure and might have diverged at an increased rate until it acquired a new but related function. Possible mechanisms leading to this new function might have been replacement of domains by insertion and/or deletion of DNA. This interpretation receives credence by the observation of "missing" residues in NGF as compared to BDNF (Fig. 3).

The extremely low divergence rate on the amino acid level for BDNF is in the range of such highly conserved proteins such as glucagon (evolutionary rate 1.2 from reptiles to mammals). The low divergence rate seems to indicate that BDNF not only fulfills a highly conserved function but also reached an "optimized" structure very early in the vertebrate lineage that tolerated only very little variation. In this context it has to be remembered that the amino acid sequences of all mammalian BDNFs determined so far are identical (Hofer et al., 1990). It is tempting to speculate if the different levels of conservation of NGF and BDNF are also reflected at the level of the corresponding receptors.

Physiological role of NGF and BDNF in fish

The available data on the expression and biological activity of the two neurotrophic factors in fish do already allow some conclusions with respect to their physiological role. BDNF mRNA was localized unambiguously in brain of adult fish; the levels of NGF mRNA were below the detection limit in all organs investigated. The localization of BDNF mRNA in fish corresponds to that in mammals where BDNF mRNA has been shown to be predominantly expressed in the CNS (Leibrock et al., 1989; Hofer et al., 1990). NGF could be detected only during a limited period of organogenesis (stage 16–24), probably reflecting its role in the formation of the fish sympathetic nervous system that takes place at this developmental time. The effects reported thus far of (mouse) NGF on fish retina neurons are to some extent contradictory. On the one hand, mouse NGF has been reported to stimulate neurite outgrowth from fish retina explants (Turner et al., 1982) and to enhance axonal regeneration following optic nerve crush (Turner et al., 1980; Yip and Grafstein, 1982). However, no evidence could be obtained for the presence of specific NGF receptors on retina ganglion neurons (Yip and Johnson, 1983). In higher vertebrates (rat, chick) no evidence for a survival action of NGF on retina ganglionic cells could be obtained (Johnson et al., 1986; Rodriguez-Tébar et al., 1989).

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